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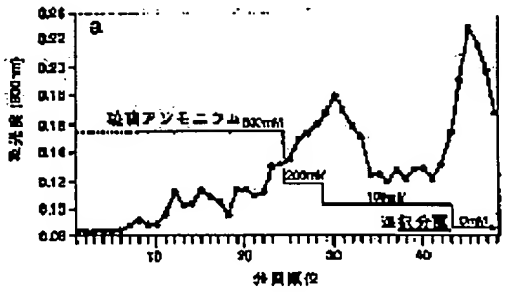
(71)Applicant: **NIPPON B C G SEIZO KK**
(72)Inventor: **KAWASHIRI KATSUHIDE**
HONDA IKURO
NAKAMURA REIKO
TOIDA ICHIRO
HAGA SHINJI
NAGAI SADAMU

(54) **METHOD FOR ISOLATING PROTEIN FROM WASTE CULTURE SOLUTION AFTER SHORT PERIOD CULTURE OF BCG BACTERIA AND MEASURING REAGENT FOR DELAYED HYPERSENSITIVITY REACTION**

(57)Abstract:

PROBLEM TO BE SOLVED: To provide a method for efficiently recover a useful protein excreted by BCG bacteria in a culture medium and provide the recovered protein as a reagent, by using a waste culture medium from a short period culture of the BCG bacteria which is discharged in large amount as an industrial waste.
SOLUTION: The subject protein is obtained by heating a waste culture medium at 40-45°C, successively filtering BCG bacteria cells and small molecular weight culture medium components to obtain a concentrated protein and carrying out an affinity purification of the protein successively by a phenylsepharose CL-4B column, a DEAE-sepharose column using urea, a sephacryl S200HR column and finally a DEAE-sepharose CL-6B column without using urea. The protein is used as a reagent.



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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[The technical field to which invention belongs] This invention relates to the reagent which makes a base resin isolated protein by the method of isolating protein more useful than the protein which BCG bacillus discharged from BCG bacillus short-term-culture liquid, and this technique.
[0002]
[Description of the Prior Art] Conventionally, the technique the technique MPB64 and MPB70 isolate from the little culture medium for five weeks or ten weeks which carried out the long term culture isolates BCG bacillus by Nagai et al. using the DEAE sephadex A-50 and DEAE Sepharose is announced by April, 1986 American society **** micro biotechnology ****** issue and in **********- and - immunity (American Society for Microbiology), Vol.52, and No.1 p 293-302. Moreover, the method of manufacturing the MPB64 above-mentioned protein using genetic engineering is learned with the JP,1-247094,A patent public presentation official report.
[0003] However, a starting material is the culture medium of a long term culture, and the technique of ** et al. and the former also has very few amounts. Moreover, after the latter thing's including MPB64 gene in a recipient and cultivating a recipient, it is the technique of taking out MPB64 from a recipient, and the operation is **** very much.
[0004]
[Problem(s) to be Solved by the Invention] This invention is the technique for separating the useful protein represented with MPB64, MPB70, etc. from the inside of the protein which reaches no less than 300 kinds which the above-mentioned biomass discharges out of a biomass, while cultivating BCG bacillus, and it is for providing a commercial scene with the reagent for delayed-hypersensitivity reaction measurement which offers and combines with a commercial scene the technique of often isolating a lot of protein of these, and makes a base resin this isolated protein.
[0005]
[Means for Solving the Problem] Using a column, load with the above-mentioned starting material and it is eluted with the buffer solution. in order to attain the above-mentioned technical probrem -- this technique invention -- the used culture medium after BCG bacillus short term culture -- a property -- things -- Carry out a fractionation to two or more pieces one by one, and the fractionation containing the protein which should be isolated from the inside of this is chosen. In the technique of the buffer solution containing the component which is different in the column refining technique of dialyzing after condensing this fractionation liquid, using the column of a different property performing the same column refining technique as the above twice [at least], and refining protein As pretreatment of the above-mentioned column refining technique, the above-mentioned used culture medium is warmed at 40 or 45 degrees C. this through a filter paper A residual BCG Tokyo bacillus is removed first, subsequently with a molecular weight of about 5000 or less protein and a culture medium component are removed a ** exception, and the above-mentioned used culture medium is condensed. subsequently An ammonium sulfate (following only AS) is added to this concentration liquid 60% at a saturation, all protein is settled, a centrifugation separates protein, and it considers as the technique of isolating protein from the used culture medium after BCG bacillus short term culture characterized by supplying this separated protein to the following process.
[0006] In the column refining technique of the second phase of the above-mentioned column refining technique of the technique of isolating protein from the used culture medium after the above-mentioned BCG bacillus short term culture in order to attain the above-mentioned technical probrem A DEAE Sepharose CL-6B column is used as a column. as buffer solution An equilibration is carried out by 500ml (pH7.5) of 30mM tris bufferss containing 3M (mol) urea. Supply the protein after pretreatment of the column refining technique, and are supplied 0 or the buffer solution added 200 mMs to the same buffer solution as the above, and the inclusion concentration of NaCl is supplied for NaCl continuous gradually sequentially from the one with slight height where concentration is lower. It is desirable that an elution speed is considered as a part for 0.5ml/, a fractionation is carried out to a part for several of the divisions, and it is characterized by being the technique of separating the above-mentioned urea in the process in which choose the fractionation containing the purpose protein, condense the fractionation protein subsequently chosen, and it subsequently dialyzes.
[0007] Moreover, in order to attain the above-mentioned technical probrem, this technique invention is the a. 1st phase (head end process).
The used culture medium after BCG bacillus short term culture is warmed at 40 or 45 degrees C. this through a filter paper A residual BCG Tokyo bacillus is removed first, subsequently with a molecular weight of about 5000 or less protein and a culture medium component are removed a ** exception, and the above-mentioned used culture medium is condensed to 1/200 or 1/350. subsequently b. column refining process which AS is added to this concentration liquid 60% at a saturation, and all protein is settled, separates protein by the centrifugation, and supplies this separated protein to the following process
[0008] A phenyl Sepharose CL-4B column (the diameter f 25mm, capacity of 80ml) is loaded with the protein by the 2nd phase 1st phase, concentration is supplied in the whole quantity with slight lowness from 450 or the one where AS concentration is higher 600ml gradually to what does not contain 10mM tris hydrochloric-acid (HCl) buffer solution containing AS at all from the thing of AS inclusion f the above-mentioned AS concentration of 500mMs in order, or

continuously, and 10ml of eluates is drawn by every one by one. From each fractionation, 50micro l of biotechnology-****** protein assay reagents is mixed to what was sampled every [10micro / l], an absorbance is measured, and it asks for an elution pattern, and by electrophoresis, the fractionation in which the protein of the target molecular weight is contained is chosen, the eluate of this fractionation is condensed, and it dialyzes further.
[0009] Using the DEAE Sepharose CL-6B column (the diameter of 15mm, capacity of 75ml) which carried out the equilibration of the liquid condensed in the 3rd-phase above-mentioned 2nd phase with 3M urea, the inclusion concentration of NaCl is continuously supplied [NaCl] for 0 or the buffer solution added 200 mMs to the 30mM tris-HCl buffer solution with slight height, an elution speed is considered as a part for 0.5ml/, and the fractionation of the eluate is carried out. Subsequently, an absorbance is measured like the 2nd phase, it asks for an elution pattern, and the fractionation containing the target protein is chosen, and subsequently, the selected fractionation protein is condensed and it considers as protein-concentrate liquid.
[0010] It is eluted with the same buffer solution as having used for the equilibration the protein-concentrate liquid obtained on the 4th phase preceding paragraph story using the Sephacryl S200HR column which carried out the equilibration with the 500ml tris-HCl buffer solution of 10mMs which contains ethylene glycol (EG) and NaCl of 0.3M 10%, and a fractionation is carried out one by one. Subsequently, an absorbance is measured like the 3rd phase, it asks for an elution pattern, and the fractionation containing the target protein is chosen, and subsequently, the selected fractionation protein is condensed and dialyzed and it considers as protein-concentrate liquid.
[0011] Using what carried out the equilibration of the DEAE Sepharose CL-6B column which does not contain a urea for the protein-concentrate liquid of the 5th phase 4th phase by 500ml (pH8.7) of the 30mM tris-HCl buffer solutions, it is eluted in the 30mM tris-HCl buffer solution (pH8.7) by the eluate which raised concentration continuously to 50mMs or 100mMs, and the fractionation of the NaCl is carried out to it one by one. Next, a fractionation is chosen by the same technique as a preceding paragraph story, it condenses and dialyzes, and the target protein is obtained. It considers as the technique of isolating protein from the used culture medium after BCG bacillus short term culture characterized by consisting of the 1st phase or the 5th phase above.
[0012] In order to attain the above-mentioned technical probrem, it may be characterized by the above-mentioned purpose protein isolated in the technique of the 1st phase of the technique of isolating protein, or the 5th phase, from the used culture medium after the above-mentioned BCG bacillus short term culture being MPB64.
[0013] In the technique of the 1st phase of the technique of isolating protein from the used culture medium after the above-mentioned BCG bacillus short term culture in order to attain the above-mentioned technical probrem, or the 5th phase It may be characterized by for the above-mentioned purpose protein to isolate being MPB59, MPB64, MPB70, MPB80, and MPB85, and being the technique of isolating at least two sorts of protein in parallel to the band between simultaneous in the 2nd phase or the 5th phase beyond the above in these.
[0014] It is the reagent for delayed-hypersensitivity reaction measurement characterized for a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 from which the above isolated invention of this object by the thing [having **ed or freeze-dried] in order to attain the above-mentioned technical probrem.
[0015] It is the reagent for delayed-hypersensitivity reaction measurement of the shape of salve characterized by having kneaded a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 from which the above isolated invention of this object, and the ointment in order to attain the above-mentioned technical probrem.
[0016]
[Function of the Invention] The used culture medium which carried out the short term culture of the BCG bacillus produced in technique invention according to claim 1 when manufacturing BCG tablet as a starting material is used. By preceding into this isolating the target protein from the inside of the protein of the varieties discharged from the above-mentioned BCG bacillus at the outside of the biomass, and warming this used culture medium at 40 degrees C or 45 degrees C When the consistency of culture medium falls and it filters with a VCF, a fluidity becomes good, BCG bacillus which remains first is removed, and there is an operation by which the protein and the culture medium component of parvus molecular weight are subsequently filtered rather than the molecular weight of the target protein for a short time.
[0017] In technique invention according to claim 2, it sets to the 2nd of a column purification method besides an operation of invention according to claim 1. Use a DEAE Sepharose CL-6B column and an equilibration is carried out by 500ml (pH7.5) of 30mM tris bufferss which contain 3M (mol) urea as buffer solution. Since the protein after pretreatment of the column refining technique is supplied, 0 or the buffer solution added 200 mMs is supplied to the same buffer solution as the above and NaCl is supplied continuous gradually sequentially from the one with slight height where concentration is lower, the inclusion concentration of NaCl The operation which combination or the impurity incorporated combines with a urea, and is removed is made in protein. Moreover, by dialyzing an eluate immediately after this phase, the above-mentioned urea is removed from an eluate, the isolated short white matter is changed or most operations which weaken activity make the operation which does not make it start.
[0018] the protein made into the purpose in invention according to claim 3 from the used culture medium which carried out the short term culture of the BCG bacillus according to others, the 1st above-mentioned phase, or the 5th phase -- purity -- at least one kind of operation to isolate is made highly [operation / of invention the above-mentioned claim 1 and given in two] Especially, two or more fractionations are independently chosen, respectively at a certain time, and the protein which should be isolated in the 2nd phase makes two or more sorts of operations from which refining which it becomes independent of for every fractionation in a subsequent phase is attained. invention according to claim 4 -- setting -- the purpose protein besides an operation of the claim 3 above-mentioned publication ****** -- MPB64 -- purity -- the operation isolated highly is made
[0019] In invention according to claim 5, as purpose protein besides an operation of the claim 3 above-mentioned publication, a fractionation is simultaneously carried out independently, respectively, the simultaneous parallel of at least two of the MPB59, MPB64, MPB70, MPB80, and MPB85 sorts are carried out after that in the 2nd phase, for every protein by which the fractionation was carried out, and the operation which isolates purity highly, respectively is made.
[0020] In invention according to claim 6, since it is the reagent for delayed-hypersensitivity reaction measurement characterized for a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 which were isolated by the above-mentioned

claim 4 by the thing [having **ed or freeze-dried], the operation which carries out a specific reaction is made to these antibodies.
[0021] Since it is the reagent for delayed-hypersensitivity reaction measurement of the shape of salve characterized by having kneaded a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 which were isolated by the above-mentioned claim 4, and the ointment in invention according to claim 7, this part Human being, By applying to the skin of animals, such as a cow, it can adhere to these skins for a long time, and the inflammation or the hardening phenomenon generated on these skins is caused by the existence of an antibody to these human beings or the above-mentioned specific protein of an animal, or the operation not to cause is made.
[0022] In invention according to claim 8, it is enabled to stick this on the skin and succeeds in the operation which the skin is not made to cause the same reaction as the above-mentioned invention according to claim 7 on it, or it does not cause.
[0023]
[Embodiments of the Invention]
**** slack my ********** ****** BCG Tokyo of a material and a BCG vaccine manufacture of technique culture medium:sow ton (Sauton) synthesis culture medium use bacillus:Japan (they are [Mycobacterium bovis BCG Tokyo and] only BCG bacillus and **** below). Surface culture was carried out at 37 degrees C without transplanting and carrying out aeration of the above-mentioned BCG bacillus to the above-mentioned sow ton culture medium. The biomass cultivated on the 8th was carried out to the vaccine manufacture, and by filtration, used culture medium after carrying out a harvest was collected, and it considered as the starting material.
Column: DEAE Sepharose CL-6B, Sephacryl S200HR, and Sepharose CL-4B (made in [********** LKB] Sweden and Uppsala) were used.
[0024] contest a Sample Concentration:millimeter pore peri - cassette system (Millipore Pellicon System) XX42PEL60 (made in [milli pore company] the U.S., MA state, and Bedford) and friend contest (Amicon) YM-3 membrane (membrane) (made in [friend contest Inc.] the U.S. and MA state) were used
[0025] As 2-dimensional electrophoresis (they are only 2D-E and **** below):2D-E, the milli pore in ********** (Milliporeinvestigator) 2-D electrophoresis system was used. The 1st dimension of the isoelectric focusing was performed using 9.5M urea containing 5.5% base and acid-radical amphoterism (******** light) mixture by 18000V [pI3 or the domain of 10, and]/hr. The 2nd ********** dodecyl and dimension (they are only SDS-PAGE and **** below) of the **********-polyacrylamide-gel (Sodium dodecyl sulphate-polyacrylamide gel) electrophoresis were performed by 16000mW using 12.5% polyacrylamide . gel (polyacrylamide gel).
[0026] Reagent: The thing for the 2-D electrophoresis systems by the millimeter pore company was used. The argentation reagent (made in [first chemicals incorporated company] Japan and Tokyo) dyed gel. Measurement of molecular weight used standard protein (the U.S., CA state, Richmond, made in a biotechnology ****** laboratory). As other reagents, all the reagents without especially notes used the thing by Wako pure medicine incorporated company.
[0027] the gestalt 1 of operation -- the gestalt of the operation which next includes technique invention according to claim 1 to 5 is explained
The 1st phase (pretreatment)
First, it carries out a ** exception, and BCG biomass which warms the above-mentioned starting-material slack used culture medium at 45 degrees C of 40 degree-C **s, filters it through the filter paper (made in [Oriental filter paper incorporated company] Japan and Tokyo) of No.2, and remains 80 or 100l. in the above-mentioned starting material in it is continuously supplied to a milli pore peri contest cassette and equipment with 40 degrees C or 45 degree-C status. Two VCFs of the equipment of this milli pore peri contest cassette PLCC00005 are used in order to remove a with a molecular weight [like a glycerol or other culture-medium components] of 5000 or less parvus molecule. Thus, the first starting material is condensed to 300 or 400ml. In order that this concentration may save time, it may be divided into a part for several of the divisions, and may be performed using two or more above-mentioned millimeter pore peri contest cassette PLCC00005.
[0028] When warmed by the domain of the temperature as above-mentioned, a consistency falls, and the above-mentioned starting-material slack used culture medium makes it easy to pass the VCF of the parvus molecule of 5000 or less molecular weight, and is useful to shortening transit time. A concentrate is further filtered again in the milli pore membrane millimeter pack 60 (0.22 micrometer millimeter pore Co., **), and removes BCG biomass completely.
[0029] The ammonium sulfate was added to the condensed incubation filtrate 60% at the saturation, and all protein was settled. After carrying out the cooling store of this precipitate mixed liquor in the **** or cold storage, it dissociates by the centrifuge method by 6000rpm, and a supernatant liquid is removed. 2D-E (2-dimensional electrophoresis) of this concentrate is shown in drawing 1 . The protein which BCG bacillus secretes is scattered as a spot which many became independent of with the isoelectric point and molecular weight. For example, the arrow head shows MPB64 (26kDa).
[0030] A phenyl Sepharose CL-4B column is loaded with the precipitate of all the proteins obtained according to the 2nd phase (affinity (affin) refining of protein by phenyl Sepharose) phase 1. up to [that in which this column does not contain ammonium-sulfate (AS) concentration at all from 500mMs] -- one by one -- 500mM AS:150ml, 200mM AS:50ml, 100mM AS:150ml, and AS: -- nothing -- it was eluted at the room temperature using the 10mM tris HCl buffer solution reduced for every phase with 120ml
[0031] It **s and 10ml of eluates is drawn by every. From each fractionation liquid, a sample is extracted every [10micro / l], 50micro (Protein Assay Bio-Rad) l of biotechnology ****** (Bio-Rad) reagents diluted 5 times with this is mixed, and the absorbance of 600nm is measured with a microplate reader. The example of the elution pattern is shown in drawing 2 . Since it is already announced that the peak of MPB64 appears in drawing 2 after the peak of the biggest protein with which MPB is contained, when refining MPB64, estimation of the fractionation in which MPB64 is contained detects MPB64 by the molecular weight which used an elution pattern and SDS-PAGE, and chooses the fractionation in which these are contained. In the 2nd phase, when one or more fractionations are covered and there is the target protein, two or more of these fractionations are chosen.
[0032] As the concrete technique of SDS-PAGE, it is 40mA in 10 or 20% polyacrylamide gel, using a multi-gel kit (made in [Japanese Daiichi Phamaceutical Co., Ltd.] Japan and Tokyo) as a gradient gel, an argentation reagent dyes protein together with standard protein, MPB64 and MPB70, and the fractionation containing standard protein and the corresponding protein

is chosen. [for example,] The fractionation containing the target protein 64, for example, MPB, is condensed through friend contest YS-3 membrane.
[0033] A concentrate is put into a slide-A-riser cassette (made in [pierced earring] the U.S., Illinois, and Rockford), and is dialyzed at 4 degrees C with the 10mM tris HCl buffer solution of pH 7.5 one whole day and night. In the dialyzed raw material, through and the amount of proteins carried out determination of the milli pore ********** GV VCF (0.22 micrometers) by the tanker method.
[0034] The 3rd phase (refining by DEAE-Sepharose column containing a urea)
The equilibration of this was carried out using the DEAE Sepharose CL-6B column (the diameter of 15mm, capacity of 75ml) by 500ml (pH7.5) of the 30mM tris-HCl buffer solutions containing 3M urea. The concentrate from the 2nd phase is supplied to the above-mentioned DEAE Sepharose CL-6B column, and NaCl (0 or 200mMs) which had a concentration gradient in the buffer solution of the same component as having used for the above-mentioned equilibration is added, and it is eluted.
[0035] 4 degrees C -- for 1 minute -- the elution speed of 0.5ml ****** -- an eluate -- respectively -- a 5ml fractionation -- 46 -- or it draws for 100 minutes By the same technique as the 2nd phase, the absorbance of 600nm is measured for each of this fractionation using a biotechnology ****** reagent. The result is as being shown in drawing 4 . Moreover, the sample of a fractionation detects the target protein 64, for example, MPB, by SDS-PAGE like the 2nd phase, chooses the fractionation corresponding to this, performs concentration, dialysis, and filtration like the 2nd phase, and obtains the target concentrate. In the process of the above-mentioned dialysis, the urea component added at the time of elution is removed. Determination also of the amount of proteins is carried out by the same technique as the 3rd above-mentioned phase.
[0036] The 4th phase (refining by the Sephacryl column)
The equilibration of the Sephacryl S200HR column (the diameter of 25mm, capacity of 450ml) is carried out with the 500ml 10mM tris-HCl buffer solution (pH7.5) which contains ethylene glycol and 0.3MNaCls 10% at low temperature. The concentrate obtained in the 3rd phase is supplied to the above-mentioned Sephacryl S200HR column. It is eluted at the elution speed of 0.5ml in 1 minute with the same buffer solution used for the above-mentioned equilibration. Carry out the fractionation of every 5ml to a fractionation container one by one 46 or 100, and BCA protein analytical reagent (made in pierced earring company] the U.S., Illinois, and Rockford) is used for the sample from each fractionation instead of the biotechnology ****** reagent of the 2nd phase. By the same technique as the 3rd phase, a 562nm spectrometry is resembled by SDS-PAGE, therefore, the target protein is detected and the fractionation corresponding to this is chosen (refer to the drawing 4).
[0037] After dyeing dyeing of the gel at this time by CBB, its thing with more high photographic sensitivity to do for the argentation is desirable. The fractionations containing the target protein are collected, and it condenses by friend contest YM-3 membrane, and dialyzes in the 10mM tris-HCl buffer solution (pH8.7) at 4 degrees C one whole day and night. Furthermore, this is filtered by the 0.45-micrometer Millipore filter. The tanker method estimates the collected amount of proteins.
[0038] The 5th phase (refining by DEAE-Sepharose column which does not contain a urea)
In the DEAE Sepharose CL-6B column which carried out the equilibration with the 30mM tris-HCl buffer solution (pH8.7) which contains NaCl of 50mMs which do not contain a urea in the culmination of refining The protein collected according to the phase 4 is supplied, and with the 500ml 30mM tris-HCl buffer solution which contains NaCl of the concentration gradient of 50 or 100mMs at 4 degrees C, it is eluted at the elution speed of 0.5ml in 1 minute, and draws 5ml at a time to minute 46 or 100 one by one (refer to the drawing 5).
[0039] A detection of the target protein is performed by the absorbance of 600nm, and SDS-PAGE by the same technique as the 3rd phase. The fractionation which contains the target protein like a preceding paragraph story is collected and condensed, and it dialyzes in the 10mM tris-HCl buffer solution (pH8.7), and filters with a milli ****** Rex-GV VCF (0.22 micrometer millimeter pore Co.). The amount of last recovery proteins is measured by the tanker method.
[0040] In the gestalt of the above-mentioned operation, mainly, although explained about the isolation of MPB64 and MPB70, the method of isolating other useful protein simultaneously is also included in invention of the above-mentioned claim 1 or the claim 5. When isolating the protein of several of these sorts, the isolation of carrying out in parallel for every protein after dividing the protein of several sorts into each in the 2nd phase is natural. That is, two or more protein will be refined in concurrency.
[0041] In the 3rd above-mentioned phase and the 5th phase, although the concentration of the additive of the buffer solution used for elution explained the thing with a concentration gradient, the technique of supplying the thing of the concentration difference of the gradual additive divided into 4 or 10 phases one by one is also included in the gestalt of this operation.
[0042] It is the gestalt of implementation of invention of the object of gestalt 2 claim 6 publication of operation, and as it is, a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 which were refined according to the gestalt 1 of the above-mentioned operation is sealed with inert gas in a bottle or ampul, and let it be a product.
[0043] What was soluble in 2ml of the phosphoric-acid buffer solutions, mixed 0.5g of hydrophilic salve, 1.5g, and tragacanth gum powder small quantity (0.02 or 0.05g) to this to 15mg of kinds of the MPB59, MPB64, MPB70, MPB80, and MPB85 which are the gestalt of implementation of invention of the object of gestalt 3 claim 7 publication of operation, and were refined according to the gestalt 1 of operation, and was sealed in the bore of 8mm, or the 11mm flat Each component, an above-mentioned weight, and above-mentioned capacity are an example, and there is no limitation-meaning.
[0044] To a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 which are the gestalt of implementation of invention of the object of gestalt 4 claim 8 publication of operation, and were refined according to the gestalt 1 of operation Melt in 2ml of the phosphoric-acid buffer solutions, and it mixes to glycerol 1ml and/or polyethylene-glycol 10%. In the above-mentioned aluminum pan with which the filter paper is put in into the strap for patch tests (Patch and test) which sticks on the skin and tests an allergic reaction, or the aluminum pan What [0045] sealed 5 or the thing which carried out 20microl sinking in at the plastic envelope or the airtight bag to the so-called fin chamber which sticks and uses for the skin the above-mentioned filter paper which hung down the antigen which consists of the above-mentioned protein, and sank in this antigen
[Effect of the Invention] In invention according to claim 1, it is the deployment of industrial waste, since it is use of the use

culture medium after BCG bacillus incubation, since the viscosity of this culture medium falls remarkably by warming the above-mentioned used culture medium at 40 degrees or 45 degrees, it becomes easy to pass along a filter paper, the processing time becomes short, ********** with the high consistency especially contained in used culture medium is removed, and future handling becomes easy. Moreover, a degradation of a proteinic potency is not caused in warming of this level.

[0046] A DEAE Sepharose CL-6B column is used in invention according to claim 2. After ****-izing by 30mM tris buffers (pH7.5) which contains 3M (mol) urea as buffer solution, gradually concentration from 0 to 200mMs for NaCl to the buffer solution containing the same urea with slight height By supplying sequentially from the one where concentration is lower, and eluting the target protein By combination or the impurity surrounded being removed by protein, and holding the neutrality of an eluate during elution, the above-mentioned urea is removed from refining protein by the dialysis just behind it, and **, and suppresses the influence which brings the irreversible denaturation by the urea to protein to the minimum extent by it.

[0047] In technique according to claim 3, the fractionation of the useful protein can be carried out to a kind or two sorts or more of protein by adopting the technique of the 2nd phase outside the above-mentioned claim 1 and an effect of the invention according to claim 2. Furthermore, it has the effect which raises the purity of each protein by the technique after the 3rd phase. The protein isolated, respectively is identified the same thing as compared with standard protein, and what also has purity of the same grade as a standard thing is obtained, respectively.

[0048] In addition, [0049] which can be applied also to refining of other mycobacteria antigens which contain other defense antigen matter contained in the above-mentioned used filtrate, tuberculin (tuberculn), the common antigen of a mycobacteria (mycobacteria), etc. although not illustrated above In invention given in claim 4 term, since the protein isolated outside the effect of the claim 1 or the claim 3 is MPB64, combining other MPB70, isolated MPB64 distinguishes tubercular infection and tubercular BCG positivity, and can judge them.

[0050] In invention according to claim 5, since the protein isolated outside an effect according to claim 1 to 4 is the technique of performing two or more sorts of protein of MPB59, MPB64, MPB70, MPB80, and MPB85 in parallel to the band between simultaneous by the technique of the 2nd phase or the 5th phase, it has the effect that it is efficient and two or more sorts of protein can be isolated effectively, respectively, from a single starting material.

[0051] In invention according to claim 6, since it is a configuration as above-mentioned and MPB59, MPB64, MPB70, MPB80, and MPB85 are sealed by the single taste, respectively, it dilutes with a proper dilution agent like the conventional tuberculin-reaction reagent, and it can be used for the skin of human being and an animal at an application or an intradermal injection. Moreover, it can also make it easy to knead with a proper hydrophilic ointment, a glycerol, a polyethylene glycol, etc., to apply to the skin as salve, and to use it as a reaction reagent. Since it is already the salve outside the effect of the invention according to claim 6, handling is easy, and it is easy to make the skin plaster in invention according to claim 7 for a long time. In invention according to claim 8, since it is the strap further outside the effect according to claim 8, it can stick on the skin as it is.

[0052] When MPB64 was isolated by the technique of the gestalt 1 example of experiment 1 operation using 100l. of the culture medium immediately after cultivating BCG bacillus for eight days, in the 5th last phase, 5mg of isolated MPBs64 was obtained. When it **ed, and MPB64 and MPB70 which were obtained performed SDS-PAGE and having been examined by the immuno blotting using anti-MPB64 and anti-MPB70 antibody, the respectively same reaction as canonicals MPB64 and MPB70 was shown.

[0053] MPB64 and MPB70 are isolated by the example 2 of an experiment, and the mode 1 of operation, respectively. The place which prepared and experimented in the guinea pig which inoculated the guinea pig and BCG bacillus which were infected with the tubercule bacillus, respectively combining two sorts of these protein, Although it was the positivity which the guinea pig which carried out the tuberculin change to positive by BCG inoculation showed the electropositive reaction to MPB64 and MPB70, and was infected with the tubercule bacillus to MPB64, to MPB70, it was negative. Tubercular infection or BCG change to positive has distinguished from the above result easily.

[0054] Example of experiment 3MPB59 has the same effect as a tuberculin reaction, and was applicable to the test of whether there is any allergy to the protein containing this about MPB80 and MPB85. It can be used more effective in decision of tubercular infection of a man and a cow than an experiment of this.

[Translation done.]

Effect

[Effect of the Invention] In invention according to claim 1, it is the deployment of industrial waste, since it is use of the use culture medium after BCG bacillus incubation, since the viscosity of this culture medium falls remarkably by warming the above-mentioned used culture medium at 40 degrees or 45 degrees, it becomes easy to pass along a filter paper, the processing time becomes short, ********** with the high consistency especially contained in used culture medium is removed, and future handling becomes easy. Moreover, a degradation of a proteinic potency is not caused in warming of this level.

[0046] A DEAE Sepharose CL-6B column is used in invention according to claim 2. After ****-izing by 30mM tris buffers (pH7.5) which contains 3M (mol) urea as buffer solution, gradually concentration from 0 to 200mMs for NaCl to the buffer solution containing the same urea with slight height By supplying sequentially from the one where concentration is lower, and eluting the target protein By combination or the impurity surrounded being removed by protein, and holding the neutrality of an eluate during elution, the above-mentioned urea is removed from refining protein by the dialysis just behind it, and **, and suppresses the influence which brings the irreversible denaturation by the urea to protein to the minimum extent by it.

[0047] In technique according to claim 3, the fractionation of the useful protein can be carried out to a kind or two sorts or more of protein by adopting the technique of the 2nd phase outside the above-mentioned claim 1 and an effect of the invention according to claim 2. Furthermore, it has the effect which raises the purity of each protein by the technique after the 3rd phase. The protein isolated, respectively is identified the same thing as compared with standard protein, and what also has purity of the same grade as a standard thing is obtained, respectively.

[0048] In addition, [0049] which can be applied also to refining of other mycobacteria antigens which contain other defense antigen matter contained in the above-mentioned used filtrate, tuberculin (tuberculn), the common antigen of a mycobacteria (mycobacteria), etc. although not illustrated above In invention given in claim 4 term, since the protein isolated outside the effect of the claim 1 or the claim 3 is MPB64, combining other MPB70, isolated MPB64 distinguishes tubercular infection and tubercular BCG positivity, and can judge them.

[0050] In invention according to claim 5, since the protein isolated outside an effect according to claim 1 to 4 is the technique of performing two or more sorts of protein of MPB59, MPB64, MPB70, MPB80, and MPB85 in parallel to the band between simultaneous by the technique of the 2nd phase or the 5th phase, it has the effect that it is efficient and two or more sorts of protein can be isolated effectively, respectively, from a single starting material.

[0051] In invention according to claim 6, since it is a configuration as above-mentioned and MPB59, MPB64, MPB70, MPB80, and MPB85 are sealed by the single taste, respectively, it dilutes with a proper dilution agent like the conventional tuberculin-reaction reagent, and it can be used for the skin of human being and an animal at an application or an intradermal injection. Moreover, it can also make it easy to knead with a proper hydrophilic ointment, a glycerol, a polyethylene glycol, etc., to apply to the skin as salve, and to use it as a reaction reagent. Since it is already the salve outside the effect of the invention according to claim 6, handling is easy, and it is easy to make the skin plaster in invention according to claim 7 for a long time. In invention according to claim 8, since it is the strap further outside the effect according to claim 8, it can stick on the skin as it is.

[0052] When MPB64 was isolated by the technique of the gestalt 1 example of experiment 1 operation using 100l. of the culture medium immediately after cultivating BCG bacillus for eight days, in the 5th last phase, 5mg of isolated MPBs64 was obtained. When it **ed, and MPB64 and MPB70 which were obtained performed SDS-PAGE and having been examined by the immuno blotting using anti-MPB64 and anti-MPB70 antibody, the respectively same reaction as canonicals MPB64 and MPB70 was shown.

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[Translation done.]

* NOTICES *

CLAIMS

[Claim(s)]
[Claim 1] Using a column, load with the above-mentioned starting material and it is eluted with the buffer solution. the used culture medium after BCG bacillus short term culture -- a property -- things -- Carry out a fractionation to two or more pieces one by one, and the fractionation containing the protein which should be isolated from the inside of this is chosen. In the technique of the buffer solution containing the component which is different in the column refining technique of dialyzing after condensing this fractionation liquid, using the column of a different property performing the same column refining technique as the above twice [at least], and refining the target protein As pretreatment of the above-mentioned column refining technique, the above-mentioned used culture medium is warmed at 40 or 45 degrees C. this through a filter paper A residual BCG Tokyo bacillus is removed first, subsequently with a molecular weight of about 5000 or less protein and a culture medium component are removed a ** exception, and the above-mentioned used culture medium is condensed. subsequently How to isolate protein from the used culture medium after BCG bacillus short term culture characterized by adding an ammonium sulfate (following only AS) to this concentration liquid 60% at a saturation, settling all protein, and for a centrifugation separating all the above-mentioned protein, and supplying this separated protein to the following process.
[Claim 2] In the column refining technique of the 2nd phase of the above-mentioned column refining technique as a column Use a DEAE Sepharose CL-6B column and an equilibration is carried out by 500ml (pH7.5) of 30mM tris bufferss which contain 3M (mol) urea as buffer solution. Supply the protein after pretreatment of the column refining technique, and are supplied 0 or the buffer solution added 200 mMs to the same buffer solution as the above, and the inclusion concentration of NaCl is supplied for NaCl continuous gradually sequentially from the one with slight height where concentration is lower. An elution speed is considered as a part for 0.5ml/, a fractionation is carried out to a part for several of the divisions, and the fractionation containing the purpose protein is chosen. subsequently How to isolate protein from the used culture medium after BCG bacillus short term culture characterized by being the technique of separating the above-mentioned urea in the process in which condense the selected fractionation protein and it is subsequently dialyzed.
[Claim 3] a. The 1st phase (head end process)
The used culture medium after BCG bacillus short term culture is warmed at 40 or 45 degrees C. this through a filter paper A residual BCG Tokyo bacillus is removed first, subsequently with a molecular weight of about 5000 or less protein and a culture medium component are removed a ** exception, and the above-mentioned used culture medium is condensed to 1/200 or 1/350. subsequently AS is added to this concentration liquid 60% at a saturation, all protein is settled, a centrifugation separates protein, and this separated protein is supplied to the following process.
b. gradual to what does not contain at all 10mM tris hydrochloric-acid (HCl) buffer solution which loads a phenyl Sepharose CL-4B column (the diameter of 25mm, capacity of 80ml) with the protein by the column refining process 2nd phase 1st phase, and contains AS from the thing of AS inclusion of the above-mentioned AS concentration of 500mMs in order -- or -- continuous -- AS concentration -- slight lowness ****** -- the whole quantity -- 450 or 600ml -- supplying -- an eluate -- one by one -- every 10ml -- From each fractionation, 50micro l of biotechnology-****** protein assay reagents is mixed to what was sampled every [10micro / l], an absorbance is measured, and it asks for an elution pattern, and by electrophoresis, the fractionation in which the protein of the target molecular weight is contained is chosen, the eluate of this fractionation is condensed, and it dialyzes further.
Using the DEAE Sepharose CL-6B column (the diameter of 15mm, capacity of 75ml) which carried out the equilibration of the liquid condensed in the 3rd-phase above-mentioned 2nd phase with 3M urea, the inclusion concentration of NaCl is continuously supplied [NaCl] for 0 or the buffer solution added 200 mMs to the 30mM tris-HCl buffer solution with slight height, an elution speed is considered as a part for 0.5ml/, and the fractionation of the eluate is carried out. Subsequently, an absorbance is measured like the 2nd phase, it asks for an elution pattern, and the fractionation containing the target protein is chosen, and subsequently, selected fractionation protein is condensed and dialyzed and it considers as protein-concentrate liquid.
It is eluted with the same buffer solution as having used for the equilibration the protein-concentrate liquid obtained on the 4th phase preceding paragraph story using the Sephacryl S200HR column which carried out the equilibration with the 500ml tris-HCl buffer solution of 10mMs containing 10% ethylene glycol (EG)0.3M and NaCl, and a fractionation is carried out one by one. Subsequently, an absorbance is measured like the 3rd phase, it asks for an elution pattern, and the fractionation containing the target protein is chosen, and subsequently, the selected fractionation protein is condensed and dialyzed and it considers as protein-concentrate liquid.
Using what carried out the equilibration of the DEAE Sepharose CL-6B column which does not contain a urea for the protein-concentrate liquid of the 5th phase 4th phase by 500ml (pH8.7) of the 30mM tris-HCl buffer solutions, it is eluted in the 30mM tris-HCl buffer solution (pH8.7) by the eluate which raised concentration continuously to 50mMs or 100mMs, and the fractionation of the NaCl is carried out to it one by one. Next, a fractionation is chosen by the same technique as a preceding paragraph story, it condenses and dialyzes, and the target protein is obtained. How to isolate protein from the used culture medium after BCG bacillus short term culture characterized by consisting of the 1st phase or the 5th phase above.
[Claim 4] The above-mentioned purpose protein is the technique of isolating protein from the used culture medium after

BCG incubation of invention according to claim 3 characterized by being MPB64.
[Claim 5] The above-mentioned purpose protein is the technique of isolating protein from the used culture medium after BCG incubation of invention according to claim 3 characterized by isolating protein from the used culture medium after BCG incubation characterized by being MPB59, MPB64, MPB70, MPB80, and MPB85, and being the technique of isolating two or more sorts of protein in parallel to the band between simultaneous in the 2nd phase or the 5th phase beyond the above in these.
[Claim 6] The reagent for delayed-hypersensitivity reaction measurement characterized for a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 which were isolated by the above-mentioned claim 4 by the thing [having **ed or freeze-dried].
[Claim 7] The reagent for delayed-hypersensitivity reaction measurement of the shape of salve characterized by having mixed a kind of the MPB59, MPB64, MPB70, MPB80, and MPB85 which were isolated by the above-mentioned claim 4, and the ointment.
[Claim 8] The reagent for delayed-hypersensitivity reaction measurement characterized by what further mixed at a glycerol 1ml rate to what melted at a rate of 2ml of the phosphoric-acid buffer solutions, and was mixed at a rate of 1g of hydrophilic ointments and 0.04g of tragacanth gum powder having infiltrated the strap for patch tests to 15mg of kinds of the MPB59, MPB64, MPB70, MPB80, and MPB85 which were isolated by the claim 4.

[Translation done.]